INACTIVATION OF BACTERIOPHAGE BY ACRIDINE ORANGE

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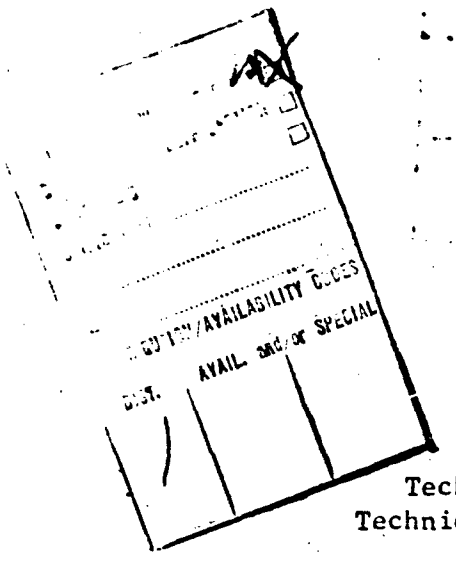

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INACTIVATION OF BACTERIOPHAGE BY ACRIDINE ORANGE

/Following is the translation of an article by V. K. Ravin, Institute of Biological Physics, USSR Academy of Sciences, Moscow, published in the Russian-language periodical Biofizika (Biophysics) Vol X, No 2, 1965, pages 261--267. It was submitted on 9 May 1964. Translation performed by Sp/7 Charles T. Ostertag Jr./.

The effect of dyes of the acridine series on extracellular viruses has been studied by many investigators /1--4/. In the event of the illumination of a mixture of virus with a dye a photodynamic inactivation of the virus takes place, following the kinetics of a reaction of the first order, but inactivation does not take place in the dark. As Yamamoto pointed out, the formation of a phage--dye complex is a reversible process and such a complex dissociates in the event of a simple dilution of the mixture /5/. Up until now the mechanism of photodynamic inactivation has not been known in detail, it is only possible to propose that inactivation is a result of the interaction of the dye with the nucleic acid of the virus /4 ; 5/. This point of view is based on the existence of a correlation between the sensitivity of the virus to the dye and the penetrability of its protein membrane for small molecules, and also on the stereochemical conformity of a molecule of the dye and the nucleotide pair in the DNA.

The present work presents data on the inactivation of bacteriophage by acridine orange taking place in the dark, which is probably a rare exception. Proof is presented that inactivation is the result of the reaction of the dye with the DNA phage. A mutant of phage λ is described which is resistant to acridine orange. The detailed mechanism of inactivation of bacteriophage by a dye is discussed.

Methods

1. Obtaining bacteriophage. An odd type of bacteriophage λ (k) was obtained by the method of ultraviolet induction. With this aim a 5-hour culture of E. coli K12(λ), incubated in the M9 synthetic medium with aeration, was exposed to an "optimal" dose and after 2 hours the bacterial fragments were removed by centrifuging at 5,000 g. The titer of the phage reached $5 \cdot 10^9$ -- $2 \cdot 10^{10}$. This phage, subsequently designated λ(k), was used for the infection of a sensitive strain E. Coli C. The E. Coli C bacteria were incubated in the M9 medium up to a concentration of $3 \cdot 10^8$, then $MgSO_4$ was added up to an end concentration of 0.01 M and phage λ(k) so that the multiplicity of infection was equal to 2--4. After 2 hours the lysate was purified by centrifuging. The titer of the phage reached $4 \cdot 10^{10}$. The phage λ(c) obtained by such a method differed from the initial phage λ(k) of the wild type, obtained by the induction of K12. These differences were conditioned by the modifications of phage DNA which are controlled by the host E. Coli C /6/.

2. Tests on inactivation. Since acridine orange is bound by the nucleic acids which are inevitably contained in the lysate, the suspension of phage used in these tests was diluted with a phosphate buffer, 0.03 M, pH 7.6, so that $\varepsilon_{260m\mu}$ was less than 0.02. 0.2 ml of such phage was added to 1.8 ml of the same buffer, in which a dilution was made, but which contained the required concentration of dye. The mixture was incubated for 10 minutes at 37°, after which a dilution was made in 0.14 M of NaCl + 5% meat-peptone broth. The number of viable particles was determined by the method of agar layers, using E. Coli C as the sensitive strain. For detecting the photodynamic effect the phage--dye mixture was poured into a Petri dish and illuminated with a 150 watt incandescent lamp (the dish was at a distance of 50--70 cm from the lamp filiment). The dye was prepared in an aqueous solution with a concentration of 200--400 γ/ml. The solutions were restored every 3--5 days.

3. Obtaining labeled p^{32} phage. The E. Coli C bacteria were incubated up to a concentration of $6 \cdot 10^7$ in a medium of the following composition: $MgSO_4$ -- 1.2 mg/ml; NH_4Ce -- 1.0 mg/ml; glucose -- 4.2 mg/ml; trisbuffer -- 20 mg/ml. The value of the pH was brought to 7.6 with the help of HCl. Radioactive potassium phosphate was added to the medium up to a concentration of 10 γ/ml, which corresponded to a specific activity of 11 millicuries/ml. The phage used for infection was diluted in 0.14 M NaCl + 0.02 M $MgSO_4$. The multiplicity of infection equaled 0.07. After 4.5 hours of incubation at 37° with aeration, the fragments of bacteria were removed by centrifuging at 6,000 g for 30 minutes (the operation was repeated), and then 5 ml of lysate was dialyzed against 3.5 literes of phosphate buffer 0.03 M, pH 7.6, for a period of 18 hours.

4. Determining the binding of the dye based on spectra. For these tests the lysate of λ(c) which was purified from bacterial fragments was treated with deoxyribonnuclease (25 γ/ml), ribonuclease (25 γ/ml), and also trypsin (10 γ/ml) for one hour in the stated sequence. The treated lysate was precipitated at 35,000 g for 1.5 hours, and the precipitate was collected in an 0.01 M phosphate buffer + 0.02 M $MgSO_4$. Treatment with enzymes was repeated again and then the phage was precipitated by centrifuging at 35,000 g. The second precipitate was resuspended in an 0.01 M phosphate buffer, pH 7.6 + 0.005 M EDTA for binding the traces of Mg^{++} ions. The resulting concentrates of λ(c) phage contained $(3 - 5) \cdot 10^{11}$ of plague-forming particles/ml, and, judging by electron-microscope pictures, were free of contamination by bacterial fragments and other extraneous particles. Measuring of spectra was done on the SF-4 spectrophotometer at room temperature.

Results

The curve of inactivation of λ(c) phage by acridine orange is shown in figure 1. It can be seen that the photodynamic effect did not have a real significance under the conditions of our experiments: Inactivation takes place practically the same in the dark as during illumination. The plateau at small concentrations of dye testifies to the fact that inactivation is a multi-hit process. Part of the population of phage, preserving viability

during treatment with doses of more than 5--10 γ/ml, is made up of a mutant which is resistant to acridine orange. Acridine orange also exerts a weakly expressed phagostatic effect: At a concentration of dye $\sim$30 γ/ml the number of bacteria infected by phage of λ(c), and producing full-value phage particles, in decreased by three-five times. A further increase in the concentration of the dye leads to the death of the cells. The mutant preserves a resistance to the dye even during intracellular development /7/.

The inactivation of λ (c) bacteriophage by acridine orange is suppressed to a considerable degree by spermine and Mg^{++} ions. In the appropriate test spermine was added to a suspension of phage, and after 20 minutes -- the dye. The defensive action of spermine is apparently not connected with its direct interaction with the dye; in the presence of spermine the spectrum of acridine orange is not changed; besides this, both compounds are equally charged at a neutral pH.

Figure 2 presents the spectra of acridine orange, bound with the λ(c) bacteriophage. With an excess of phage ($> 5 \cdot 10^{11}$ of spot forming particles/ml + 5 γ/ml of dye) the spectrum of the dye has a maximum at λ = = 502 mμ. With the decrease in the concentration of phage a characteristic change of the spectrum takes place, and at $5 \cdot 10^{10}$ of plague-forming particles/ml + 5 γ/ml the spectrum of the bound dye has a maximum at λ = 465 mμ. Since the number of plague-forming particles does not correspond with the number of particles which are binding the dye, and also based on the reason that phage DNA in the phage particle is surrounded by protein, the described changes in the spectrum of the dye cannot be quantitatively compared with the spectra of the DNA--dye complexes. However, the presence of two maximums in the absorption spectra of the dye bound with phage agrees qualitatively with the results of Bradley and Wolf, who observed such modifications in the spectrum of acridine orange in the presence of DNA /8/. Besides this, analogous results were obtained by us for the phage λ DNA--acridine orange complex.

From spectral measurements it is possible to determine the number of molecules of dye which one phage particle can bind. However, this may be done more accurately if the free dye is removed by centrifuging. For this the dye was added to the suspension of phage up to "saturation", that is, until in the spectrum of the bound dye, a clear maximum appears at 494 mμ, corresponding to the free dye. The mixture was centrifuged at 35,000 g for 1.5 hours. The precipitate was resuspended in a phosphate buffer, pH 7.6. All the dye in the precipitate turned out to be bound, and the maximum of the adsorption spectrum was at λ = 502 mμ. Following the second centrifuging of this solution the supernatant did not contain the dye. It was found that one phage particle was capable to irreversibly bind $\sim 5 \cdot 10^4$ molecules of dye, which is close to the number of nucleotide pairs in a molecule of phage λ DNA.

In the event of photodynamic inactivation of bacteriophage by dyes, Yamamoto showed that the formation of the phage--dye complex is a reversible process and such a complex dissociates during a simple dilution of the mixture

and during subsequent illumination photodynamic inactivation does not take place /5/. As can be seen from what has been said above, in our case simple dilution did not disrupt the phage--dye complex (at any rate not fully). However, the phage may be fully released from the dye. When the pH of the medium is raised up to 10.7 the acridine orange transforms into an alkylated form and loses the capability to be bound by nucleic acids. Under these conditions the absorption spectrum of the dye has a maximum at λ 440 mμ /9/. In such an experiment KOH was added to the phage--dye mixture in an 0.005 M phosphate buffer and the extinction ratio was determined at λ 465 mμ and 440 mμ. This value served (only in comparisons) as the measure of the binding ability of the dye. At the same time a determination was made of the restoration of the biological activity of the γ (c) phage which was inactivated by the dye (20 γ/ml). For this, the suspension of inactivated phage was diluted 10 times with a medium with a given pH, and this dilution was incubated for 10 minutes at room temperature, after which dilutions by 100 and 1000 times were made in the same medium in which the dilutions were made in the tests on the inactivation of phage. As is seen from table 1, the transition of the dye into the alkylated form takes place also for the phage bound dye, but this is not accompanied by the restoration of the biological activity of the inactivated phage.

In order to verify if there are significant impairments to the protein membrane of the phage following treatment with acridine orange, a determination was made of the adsorption capability of the λ(c) phage, inactivated by the dye. E. coli C bacteria, which were found in the middle of the logarithmic phase of growth, were infected with P^{32} labeled λ(c) phage. Another part of the culture was infected with the same phage, but it was preliminarily treated with a dose of 20 γ/ml of acridine orange. Adsorption was carried out in meat-peptone broth for 15 minutes at 37°. After completion of the adsorption the bacteria were washed off twice by cold centrifuging. Adsorption of the non-inactivated phage was determined by the decrease in the number of plague-forming particles in the supernatant liquid following the first centrifuging, and also by the number of infected centers and lysogenic cells in the second precipitate. In the capacity of a control the same amount of labeled phage was taken, but it was added to pure broth. The average results of several tests of such a type are presented in table 2, from where it is seen that the binding of radioactive phosphorus with bacteria is the same, both for intact as well as for phage inactivated by dye. On the basis of this it can be concluded that the adsorption properties of λ(c) phage do not change following its inactivation by dye.

Though the λ (c) phage inactivated by dye is also adsorbed on sensitive bacteria, the concentration of viable bacteria of E. coli C does not change following adsorption of two-thirds of the phage particles on the cell. As a result of the adsorption of intact phage, under these conditions the concentration of viable E. coli C bacteria is cut in half, and a large part of the surviving ones turned out to be lysogenic (table 3).

Discussion

The above noted qualitative analogy between the spectra of the phage-

-dye complex and the spectra of the DNA--dye complex makes it possible to propose that the interaction of the dye with the phage is in essence an interaction of the dye with phage DNA. The result of the test on the adsorption of inactivated phage makes it possible, on the other hand, to propose that the protein membrane of phage is not harmed by the dye. It is true, there is no guarantee of the fact that the dye does not harm protein structures which are not bound with the process of adsorption, but nevertheless are necessary for the injection of DNA, etc. All the same it seems to us that this possibility is unlikely. It is difficult to imagine that acridine dyes, which in general react weakly with proteins, would cause the selective inactivation of any phage proteins while preserving the function of others. It is most probable that the inactivation of λ phage by acridine orange takes place as a result of the interaction of the dye with phage DNA, and not with the protein.

The fact that phage inactivated by dye does not exert a destructive action on the cell does not contradict the conclusion drawn, because phage inactivated by ultraviolet is also devoid of this effect, though in this case it is known that the phage DNA is harmed. If the assumption is made that the reaction of phage with the dye is accompanied by damage to the DNA, then this result (table 3) testifies that the harm referred to is sufficiently profound. Apparently, in the bacteria which was adsorbing the inactivated λ (c) phage even an incomplete synthesis of the phage, which would lead to cell death, does not take place.

The protective action of spermine which we detected is explained most naturally of all by the fact that it blocks the negatively charged phosphate groups of the phage DNA and creates a steric obstacle for the admission of the molecules of the dye to it. However, the dye is a more active rival: Three to five times higher doses of spermine are required for "protection" from a specific dose of dye.

An analogous result was obtained by Kay in the case of the inhibition of the intracellular development of T3 phage under the influence of proflavine /10/. Besides this, spermine also exerts a "protective" effect in vitro, by protecting the phage λDNA from breaks under abrupt hydrodynamic influences /11/. These data also testify in favor of the assumption concerning the ability to be damaged on the part of phage DNA during inactivation of the phage by the dye.

The previous inactivation of phage by the dye is apparently nonreversible. The removal of the dye does not restore the biological activity of the inactivated phage (see table 1). It is true that a guarantee could not be given that the dye was completely removed, however, it is most probable that inactivation takes place as the result of the irreversible damage to the molecule of phage DNA, and the absence of "restoration" is connected mainly with the irreversibility of this damage, and is not the result of the incomplete removal of the dye during an increase of the pH of the medium up to 10.7.

Though the very case of the dark inactivation of phage by the dye is exceptional, there are no bases to think that the stand concerning the ability to be damaged on the part of the phage DNA is not related also to the process of photodynamic inactivation.

Conclusions

1. A case has been described of the dark inactivation of λ phage by acridine orange. Dark inactivation is possible in solutions with a low ionic strength at a weakly alkaline pH. Inactivation follows the kinetics of a multi-hit process.

2. The spectrum of the dye in the presence of λ phage is changed in the same manner as the spectrum of the dye is changed in the presence of DNA.

3. The λ phage inactivated by the dye preserves the adsorption capability.

4. The formation of the phage--dye complex, which leads to dark inactivation, is an irreversible process. Such a complex does not dissociate upon removal of the free dye. Apparently, injury to the sensitive structure is also irreversible: Removal of the dye does not restore the biological activity of the phage.

5. The data presented testifies that the sensitive structure is the phage DNA and not the protein membrane.

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Literature

a. Welsh, J. N., Adams, M. H., J. Bacteriol. 68, 122, 1954

b. Hiatt, C. W., Trans. N. Y. Acad. Sci. USA, 23, 66, 1960

c. Crowther, D., Melnick. J., Virology 14, 11, 1961

d. Gendon, Yu. Z., Vopr. virusol. 1, 16, 1964

e. Yamamoto, N., J. Bacteriol. 75, 443

f. Ravin, V. K., "Thesis of Reports at the All-Union Symposium on Experimental Mutagenesis of Animals, Plants and Microorganisms, Moscow, 1965

g. Bradley, P. F., Wolf, M. K., Proc. Nat. Acad. Sci., 45, 944, 1959

h. Kay, D., Biochem. J. 73, 149, 1959

i. Kaiser, A. D., J. Mol. Biol. 6, 141, 1963

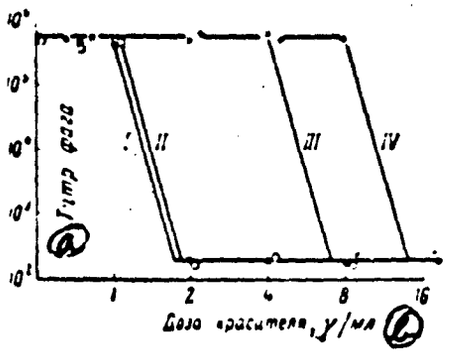

Figure 1. Kinetics of inactivation of λ(c) bacteriophage by acridine orange.
a. Titer of phage, b. Dose of dye, γ/ml.
1. Inactivation in the dark; 2. Same with illumination; 3. Same following preliminary incubation in a solution with 30 γ/ml of spermine; 4. Same following preliminary incubation in a solution with 80 γ/ml of spermine. The mixture of phage with spermine was added to an equal volume of a solution of the dye.

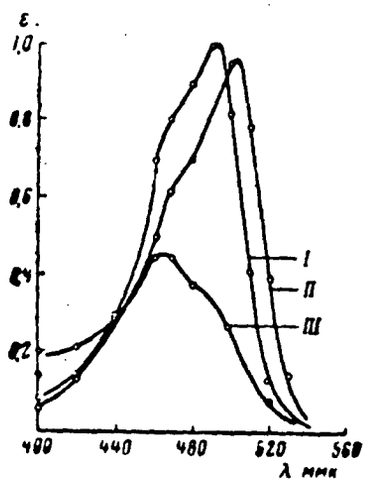

Figure 2. Spectra of the dye bound by bacteriophage.
III - 5 γ/ml of acridine orange + $1 \cdot 10^{11}$ particles of phage/ml; II - 5 γ/ml of dye + $1 \cdot 10^{12}$ particles of phage/ml; I - 5 γ/ml of free dye.

Table 1

Dissociation of the phage--dye complex with an increase of the pH of the solution

	pH			
$\varepsilon_{465}/\varepsilon_{440}$	7.6	8.4	10.3	11.4
AO	2.36	2.03	1.03	0.55
λ(c)	1.41	1.38	1.22	0.74
Titer of phage				
Inactivated λ(c)	$1 \cdot 10^3$	$1.5 \cdot 10^3$	$1.3 \cdot 10^3$	$1.4 \cdot 10^3$
Activated λ(c)	$1.7 \cdot 10^6$	$1.7 \cdot 10^6$	$2.0 \cdot 10^6$	$1.5 \cdot 10^6$

Table 2

Adsorbtion of bacteriophage, inactivated by acridine orange, on E. coli C. Description of the experiment is in the text.

	E. coli C + λ(c)	E. coli C + λ(c) (inactivated)	Control
Initial test			
Concentration of bacteria	$3 \cdot 10^8$	$3 \cdot 10^8$	-
Titer of phage	$1 \cdot 10^8$	$1 \cdot 10^3$ *	
Radioactivity, imp/min	5000	4900	10,000
Titer of phage in supernatant liquid	$4 \cdot 10^6$		
Precipitate after second centrifuging		-	-
Concentration of bacteria	$1.2 \cdot 10^3$	$1.1 \cdot 10^8$	-
Number of infection centers	$2 \cdot 10^7$	-	-
Radioactivity, imp/min	248 ± 14	257 ± 16	2 ± 10
Adsorbtion, %	96	92 ± 5 **	-
Precipitable part P^{32}, %	5.1	4.9	<0.2

* Titer before treatment with dye

** Calculated according to binding of P^{32}

Table 3

Interaction of phage, inactivated by dye, with E. coli C.

	I	II	III	IV
Concentration of bacteria	$1.5 \pm 0.1 \cdot 10^8$	$1.5 \pm 0.1 \cdot 10^8$	$1.5 \pm 0.1 \cdot 10^8$	$1.5 \pm 0.1 \cdot 10^8$
Titer of phage	-	$5 \cdot 10^8$	$5 \cdot 10^8$ *	-
Adsorbtion, %	-	85	80 **	-
Concentration of bacteria after adsorbtion	$1.5 \pm 0.2 \cdot 10^8$	$7 \pm 1 \cdot 10^7$	$1.4 \pm 0.2 \cdot 10^8$	$1.6 \pm 0.2 \cdot 10^8$

* Before treatment with acridine orange
** Determined according to binding of P^{32}

Notes: I - 4.5 ml of culture + 0.5 ml of buffer; II - 4.5 of culture + 0.5 ml of intact λ (c) phage; III - 4.5 ml of culture + 0.5 ml of phage, inactivated by15 γ/ml of acridine orange; IV - 4.5 ml of culture + 0.5 ml of a solution of acridine orange 15 γ/ml.